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ORIGINAL PAPER

Predictive Thermal Inactivation Model for Effects and Interactions of Temperature, NaCl, Sodium Pyrophosphate, and Sodium Lactate on *Listeria monocytogenes* in Ground Beef

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Abstract The effects and interactions of heating temperature (60 °C to 73.9 °C), salt (0.0 % to 4.5 %*w*/*v*), sodium pyrophosphate (0.0 % to 0.5 %*w*/*v*), and sodium lactate (0.0 % to 4.5 %*w*/*v*) on the heat resistance of a five-strain mixture of *Listeria monocytogenes* in 75 % lean ground beef were examined. Meat samples in sterile filtered stomacher bags were heated in a temperature controlled waterbath to determine thermal death times. The recovery medium was tryptic soy agar supplemented with 0.6 % yeast extract and 1 % sodium pyruvate. Weibull survival functions were employed to model the primary survival curves. Then, survival curve-specific estimated parameter values obtained from the Weibull model were used for determining a secondary model. The results indicate that temperature and salt have a large impact on the inactivation kinetics of *L. monocytogenes*, while sodium lactate (NaL) has an impact in the presence of salt. The model presented in this paper for predicting inactivation of *L. monocytogenes* can be used as an aid in designing lethality treatments meant to control the presence of this pathogen in ready-to-eat products.

Keywords *Listeria monocytogenes* · NaCl · Sodium pyrophosphate · Sodium lactate · Thermal inactivation · Predictive model

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Introduction

Adequate thermal processes destroy *Listeria monocytogenes* and are the most effective means of guarding against the potential hazards associated with the pathogen and ensuring the safety of cooked meat products. Many factors influencing the heat resistance of *L. monocytogenes* have been documented, including the variation among strains; previous growth conditions; initial population; exposure to heat, acid and other stresses; composition and pH of the heating menstruum; as well as recovery medium and incubation conditions used for detection of survivors (Doyle et al. 2001; Juneja 2001). Inadequate heat treatments may result in an unsafe product and too much heat can adversely affect the quality. Accordingly, information from complex

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multiple factorial experiments in which the effects and interactions of several levels of factors are assessed can provide information that will lead to increased efficacy of heat treatments involving individual factors, such as heat treatment, pH, and additives etc., on destroying foodborne pathogens. Subsequently, inactivation kinetics or thermal death models are developed to predict the effects of variations in these factors on the thermotolerance of the pathogen. Models enable food processors to design optimal or reduced thermal processes for the production of microbiologically safe food with extended shelf-life. Such processed foods give home-made appeal to consumers since the reduced level of heat employed minimizes the negative impact on product quality and retains the desirable organoleptic attributes of foods.

The objective of this study was to quantitatively assess the effects of various intrinsic parameters in foods on thermal inactivation of *L. monocytogenes*. Specifically, the aim was to assess the effects and interactions of heating temperature, salt (NaCl), sodium pyrophosphate (SPP) and sodium lactate (NaL) on the thermal inactivation kinetics of *L. monocytogenes* in a 75 % lean beef. The data were subsequently used to develop a predictive inactivation model.

Materials and Methods

Bacterial Strains and Culture Preparation In this study, the following five strains of *L. monocytogenes* were used: Scott A, H7762, MF27137, MF38521, and MF46869. These were clinical, hot dog outbreak, steer/heifer, ground chicken, and pork sausage isolates, respectively. The stock cultures of these strains were maintained at −70 °C in cyrovials (Microbank™, Austin, TX) containing tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks, MD) supplemented with 10 % (*v*/*v*) glycerol (Sigma-Aldrich Co., St. Louis, MO). The cultures were activated by transferring 100 μl of the thawed culture into 10 ml of brain heart infusion broth (BHI, Difco) in 50 ml tubes and incubating for 24 h at 37 °C. These cultures were maintained in BHI at 4 °C and sub-cultured in BHI on a biweekly basis.

For obtaining late stationary phase cells, cultures were grown for 18 h at 37 °C in 50 ml of BHI, in 250 ml flasks. On the day of the experiment, each overnight culture was dispensed in sterile 50 ml centrifuge tubes and centrifuged (5,000×*g*, 15 min, 4 °C). The supernatant was discarded, the pellet was washed twice in 0.1 % (*w*/*v*) peptone water (PW) and re-suspended in PW to a target level of about 8–9 $\log_{10}$ cfu/ml. The bacterial population in each cell suspension was determined by spiral plating (Autoplate 4000 Spiral Plater, Spiral Biotech, Gaithersburg, MD, USA) appropriate dilutions in 0.1 % PW, in duplicate, onto tryptic soy agar (TSA; Difco) plates. Before inoculation in meat, a five-strain mixture of *L. monocytogenes* (ca. 8 $\log_{10}$cfu/ml) was prepared by combining equal volumes of each culture in a sterile conical vial.

Meat Preparation and Inoculation Raw ground beef (75 % lean), used as the heating menstruum, was obtained from a local grocery store. The meat was divided into 200-g portions and then mixed thoroughly with NaCl (0–4.5 %, *w*/*v*), sodium pyrophosphate (SPP; 0–0.5 %, *w*/*v*), and/or sodium lactate (NaL; 0–4.5 %, *w*/*v*). Fifty-gram portion of each treated sample was weighed into PrimeSource 8×12 vacuum pouches (BUNZL-Koch Supplies, Kansas City, MO) and vacuum-sealed. Thereafter, five of these bags were placed in barrier pouches (Bell Fibre Products, Columbus, GA), vacuum-sealed, frozen at −40 °C and irradiated (25 kGy) to destroy indigenous microflora. Irradiation was performed using a self-contained ^{137}Cs Irradiator (Lockheed Georgia Co., Marietta GA) at the Eastern Regional Research Center, ARS, USDA, Wyndmoor, PA.

Fifty-grams of thawed, irradiated ground meat was aseptically inoculated with 0.1 ml of the cocktail of five strains of *L. monocytogenes*. The inoculated samples were pummeled with a Seward laboratory stomacher 400 (UK) for 5 min to ensure even distribution of the organisms in the meat sample. Three-gram ground meat samples were then weighed aseptically, in duplicate, into sterile filtered stomacher bags (BagPage^{+}, Interscience Laboratories Inc., Rockland, MA). Negative controls consisted of meat bags inoculated with 0.1 ml of 0.1 % (*w*/*v*) PW with no bacterial cells. Thereafter, the bags were massaged manually for 30 s, flattened to a uniform thickness of not more than about 1 mm thick, and then heat-sealed. Measurements of levels made immediately before heat treatment served as the initial levels at time=0.

Experimental Design A fractional factorial design was employed to assess the effects and interactions of heating temperature (60 °C, 65 °C, 71.1 °C, and 73.9 °C), NaCl (0.0 %, 1.5 %, 2.5 %, 3.0 %, and 4.5 %*w*/*v*), SPP (0.0 %, 0.15 %, 0.3 %, 0.4 %, and 0.5 %*w*/*v*), and NaL (0.0 %, 1.5 %, 2.5 %, 3.0 %, and 4.5 %*w*/*v*). Experiments were conducted for 39 different combinations of temperatures, NaCl, SPP, and NaL levels (Table 1). A combination was designated by identifying the 4-tuple set of values of Temp, NaCl, SPP, and NaL, in this order. For the combination (65 °C, 2.5 %, 0.30 %, 2.5 %), there were 9 survival curves in 5 pairs of experiments (one of the experiments for one of the pairs was not conducted). For each of the other 38 combinations, there was one pair of experiments. In total, there were 85 survival curves covering the 43 experimental combinations (39 distinct), each with 2 replicates, with the exception of the one mentioned above; from these experiments, there were 1,479 results, with two measurements per

Table 1 Experimental design, providing the experimental combinations, number of replicates and number of times per experiment for which measurements were made

Exp ID	Temp (°C)	Salt %*w*/*v*	SPP %*w*/*v*	NaL %*w*/*v*	Number replicates	Average number "times"
1	60	1.5	0.15	1.5	2	7.5
2	60	1.5	0.15	3	2	8
3	60	1.5	0.4	1.5	2	12.5
4	60	1.5	0.4	3	2	12.5
5	60	2.5	0.3	2.5	2	15.5
6	60	3	0.15	1.5	2	9.5
7	60	3	0.15	3	2	9.5
8	60	3	0.4	1.5	2	10
9	60	3	0.4	3	2	11
10	65	0	0.3	2.5	2	8
11	65	1.5	0.3	2.5	2	8
12	65	2.5	0	2.5	2	10
13	65	2.5	0.15	2.5	2	10
14	65	2.5	0.3	0	2	8
15	65	2.5	0.3	1.5	2	7.5
16	65	2.5	0.3	2.5	9	8.3
17	65	2.5	0.3	4.5	2	8
18	65	2.5	0.4	2.5	2	10
19	65	2.5	0.5	2.5	2	10
20	65	3	0.3	2.5	2	8
21	65	4.5	0.3	2.5	2	8
22	71.1	1.5	0.15	1.5	2	10
23	71.1	1.5	0.15	3	2	8
24	71.1	1.5	0.4	1.5	2	7.5
25	71.1	1.5	0.4	3	2	8.5
26	71.1	2.5	0.3	2.5	2	8
27	71.1	3	0.15	1.5	2	8
28	71.1	3	0.15	3	2	8
29	71.1	3	0.4	1.5	2	8
30	71.1	3	0.4	3	2	10
31	73.9	0	0	0	2	4
32	73.9	0	0	4.5	2	8
33	73.9	0	0.5	0	2	5.5
34	73.9	0	0.5	4.5	2	8.5
35	73.9	2.5	0.3	2.5	2	10.5
36	73.9	4.5	0	0	2	7
37	73.9	4.5	0	4.5	2	7
38	73.9	4.5	0.5	0	2	7.5
39	73.9	4.5	0.5	4.5	2	7

designated time (with one exception because of laboratory error), used for determining the model.

Validation of Experimental Design The convective heat transfer coefficient (h) of the water bath is a measure of how well and rapidly the water is mixed. To determine the convective heat transfer coefficient during heating and cooling, a 174-mm by 90 mm by 5 mm aluminum block was used. A 3-mm diameter hole was drilled into the core and a thermocouple was placed inside, to measure the

temperature versus time during heating and cooling. The convective heat transfer coefficient was then calculated using a lumped parameter analysis and using a line of best fit for the natural logarithm of non-dimensional temperature versus time. From the results of the aluminum block, the convective heat transfer coefficient during heating and cooling were 1,200 and 1,500 W m^{-2} K^{-1}, respectively. The coefficient of determination, R^2, for each was 0.96 and 0.85, respectively.

The temperature profile versus time for the stomacher bags was calculated using a 1D transient heat conduction equation (Eq. 1) on a 1-mm-thick slab (Fig. 1) in COMSOL Multiphysics 4.3, a finite element solver.

$$\rho Cp\frac{\partial T}{\partial t} = k\frac{\partial^2 T}{\partial x^2} \tag{1}$$

where the thermal conductivity (k), density (ρ), and specific heat (Cp) values were set equal to 0.4 W m^{-1} K^{-1}, 1,020 kg m^{-3} (Pan and Singh 2001), and 2,610 J kg^{-1} K^{-1} (Sheridan and Shilton 2002), respectively. The initial temperature was 20 °C with convective heat flux boundary conditions, $-kdT/dx = h(T - T_\infty)$, that used the convective heat transfer coefficient listed above, and the ambient temperature was equal to the bath temperature. The simulation results show that the "come up" time is approximately 5.0 s. The "come up" time for this work was defined as when the dimensionless temperature, $(T_\infty - T_{avg})/(T_\infty - T_{initial})$, reached 0.05. From Murphy et al. (2004), assuming that the survival curve of microbial levels versus times is log-linear and the thermal death curve of D values versus temperature is log linear, the D value (time needed to obtain a tenfold reduction of number of cells) at 65 °C ($D_{65°C}$) and z value (temperature increase for which there is a tenfold decrease of D values) of *L. monocytogenes* in ground beef were 84.6 s and 6.01 °C, respectively. Based on these results and the two assumptions of log-linearity, $D_{71.1°C}$ and $D_{73.9°C}$ values are 8.2 s and 2.8 s, respectively. These estimates are uncertain, and are only suggestive. Therefore, due to the bags being quite thin, the effect of temperature come up time is likely to be insignificant for all bath temperatures except the highest (73.9 °C). Because all data used in our study were at times not less than 25 s, the effect of temperature come up time on the results is expected to be insignificant. Figures 2a–c show the effect that the bag thickness, convective heat transfer coefficient (how well the water bath is mixed), and thermal properties of the meat have on the average bag temperature versus time. Figure 2d shows the sensitivity of the come up time to experimental design parameters with the conclusion that the sample thickness most significantly affects the come up time.

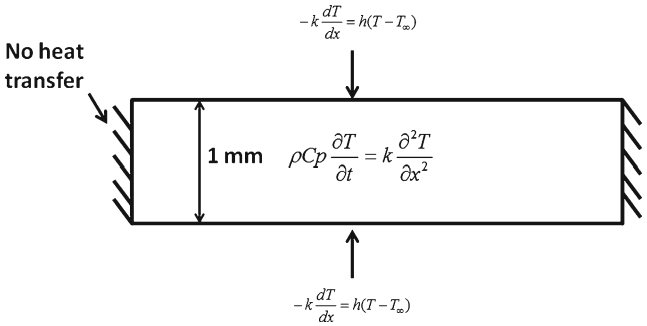

Fig. 1 Simulation schematic

The major benefit of this approach to experimental design is that the results of the simulation show what most experimental temperature measurements would not. If a temperature probe had a diameter equal to or greater than the thickness of the bag, it would only experience the thermal resistance of the thin plastic bag, therefore making the come up time almost instantaneous. But, the simulation shows that this is not the case, as there is a small thermal resistance in the 1 mm sample of meat, leading to a few seconds of come up time that can only be observed with a temperature probe on the scale of 1 μm.

Thermal Inactivation and Bacterial Enumeration Meat samples were heated in a temperature controlled water bath (Neslab RTE 17 Digital One, Thermo Electron Corp, Newington, NH) maintained at 60 °C, 65 °C, 71.1 °C, or 73.9 °C as described in our previous study (Juneja et al. 1997). Total heating times were based on the heating temperature and ranged from 150 min at 60 °C to 4 min at 73.9 °C. After two bags for each replicate were removed from the water bath at no less than 25 s, they were immediately plunged into an ice cold water bath for 2 min. Surviving microbial population densities were enumerated by surface plating appropriate dilutions, in duplicate, onto TSA supplemented with 0.6 % yeast extract (Difco) and 1 % sodium pyruvate (Sigma-Aldrich), using a spiral plater.

Control meat samples were not inoculated with *L. monocytogenes* cocktail and were plated. Also, when increased sensitivity was required, 0.1 and 1.0 ml of the undiluted suspension were surface plated. All inoculated plates were incubated at 30 °C for at least 48 h and then, bacterial colonies were enumerated. Two plates per sample were analyzed and the number of surviving CFU/ml was converted to log numbers before statistical analysis.

Statistical Methods Following normal procedures, we derive our model in two stages. The first stage is the primary model that estimates lethalities for each of the 85 experimental combinations, using the same functional form for the 85 survival curves. Then, a secondary model for predicting values of primary model parameters for arbitrary temperatures, salt, SPP, and NaL values within the range of our experimental values was developed using the estimated values of the primary model parameters for the survival curves.

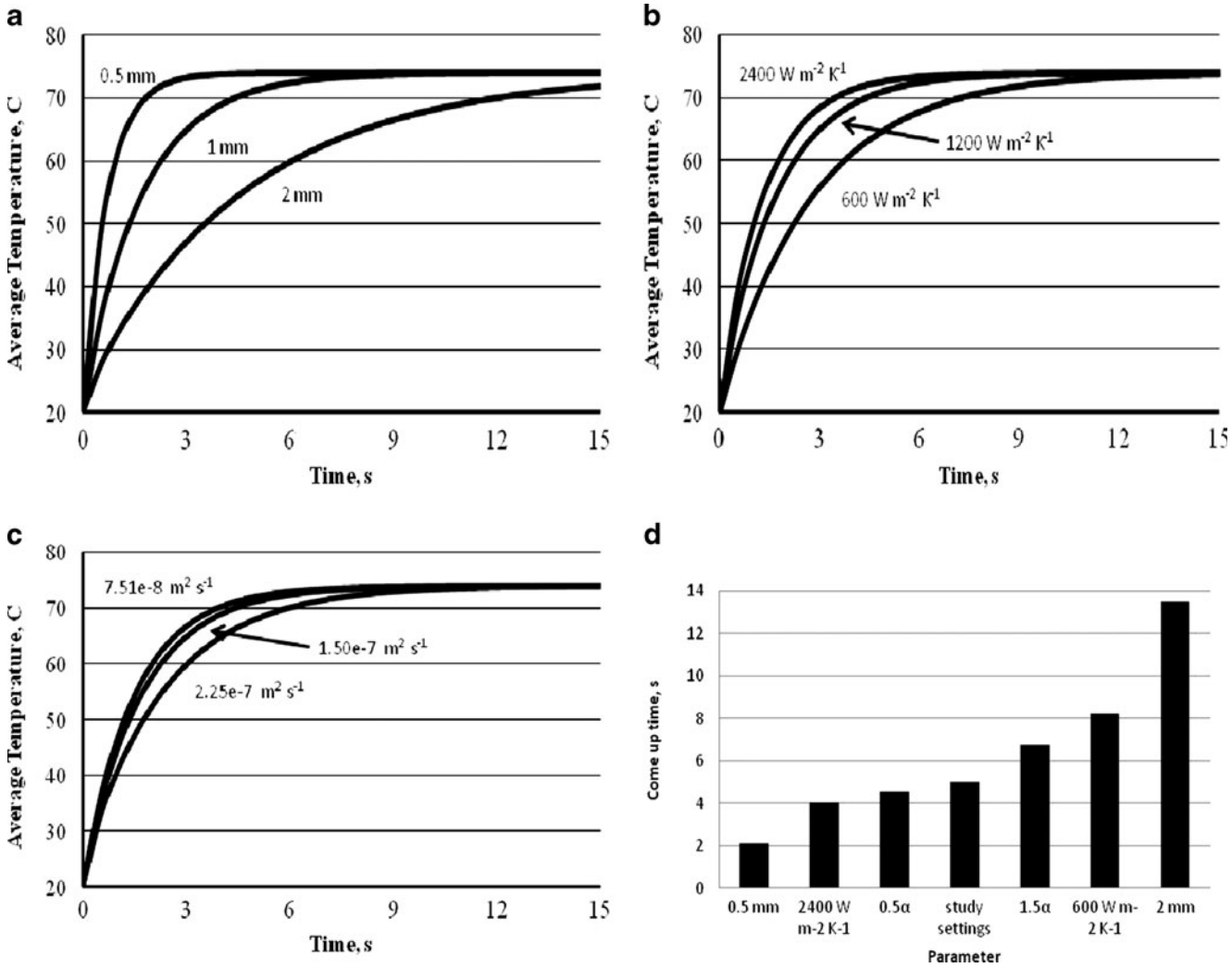

Fig. 2 **a** Effect of sample thickness on average temperature. **b** Effect of convective heat transfer coefficient on average temperature. **c** Effect of thermal diffusivity ($\alpha=k/\rho Cp$) on average temperature. **d** Effect of experimental design parameters (thermal diffusivity, heat transfer coefficient, and sample thickness) on come up time. Bath temperature was 73.9 °C

Primary Model Plots of the log of the estimated level (cfu/ml) versus time were examined to determine in general shapes of the survivor curves. Many of the curves appeared not to be log-linear. There were curves that displayed shoulder effects (concave) and others, tailing effects (convex), but few displayed both characteristics. For each survival curve, log-linear and Weibull models were compared to determine the statistical significance of a non-linear model. Thereafter, mixed-effect Poisson regression was used, where it was assumed that a plate count distribution be a Poisson distribution. Specifically, for each survival experiment, primary survival curves were assumed:

$$\begin{aligned} \mathrm{Log}_{10}(N(t)) &= n_0 - f(t|\eta) + \varepsilon \\ N(t) &= \lambda(t)/r \\ x(t) &\sim \mathrm{Poisson}\,(\lambda(t)) \end{aligned} \tag{2}$$

where: x(t) is the observed sample count at time t; r is a factor that transforms plate counts to levels (CFU/ml) and depends on the dilution and the fractional portions of the plates that were used in order to get countable numbers of colony forming units for the sample; n_0 is a constant representing the log initial level at t=0; $f(t|\eta)$ is a function of time and η, is a vector of parameters, whose values were estimated from data; $\lambda(t)$ is the expected number of CFU's for the sampled portion; and ε is an error term representing the between-sample error, assumed to be normally distributed with zero mean and standard deviation σ_r. For the linear model, $f(t|\theta)=t/\theta$, where θ is a positive constant (equal to the D-value). For the Weibull model, $f(t|\theta,\gamma)=(t/\theta)^{\gamma}$, where θ and γ are positive constants. The γ parameter commonly is referred to as the shape parameter and the θ parameter commonly is referred to as the location parameter of the Weibull. When $\gamma>1$ ($\gamma<1$), the survival curve is

concave (convex) because the second derivative of Log(N(t)) with respect to t is negative (positive). The likelihood ratio test, equal to minus 2 times the natural log of the ratio of the likelihoods at the model-specific maximum likelihood estimates, was used for determining the significance of γ being greater (lesser) than 1, and declared significance when the two–sided p-value was less than 0.05. Chi-square test values for individual data points were computed by calculating the square of the difference between the observed number and the predicted numbers of CFUs for a sample, divided by the predicted number of CFUs.

Secondary Model The estimated parameter values of the Weibull primary survival curves were logarithmically transformed. These transformed values were used as dependent variables, fitting a quadratic response surface as a function of temperature, NaCl, SPP, and NaL as independent variables. Thus, our model does not permit interaction terms involving any combination of three of these variables. For the natural logarithm of location parameter, ln(θ), linear terms of temperature and salt were forced to be in the model.

There is concern of including terms in the secondary model that do not contribute to obtaining good predictors of primary-model parameter values. Using a small significance level for inclusion of terms in the secondary model helps to prevent incorrect terms from being included in the model. Requiring too small significance, on the other hand, could prevent important variables from being included in the model thus causing poor predictions. Spurious results, those that are highly inconsistent with the other results or otherwise have a large impact of the estimated values of model parameters, can have an adverse impact on model variable selection. To aid in ensuring a good model by eliminating spurious data points, studentized residuals and DFFITS statistics were examined. Poorly fit data points were deleted when studentized residuals were greater than 3 in absolute value. For the DFFITS statistic, a common value that must be exceeded before considering a data point as influential, and thus for possible deletion, is $2(p/n)^{1/2}$ (Belsey et al. 1980) where p is the number of fixed parameters in the model and n is the number of data points. For the model, p is not larger than 5 and n is 85. Instead of 2, a coefficient of 4 was used so as to be more conservative regarding what we consider as being influential. With p=5 and n=85, $4(p/n)^{1/2}=0.97$, or about 1. Thus, before a data point would be considered influential, its DFFITS value had to be greater than 1. Interpretation of such deletions is that either there was a misspecification of the nominal independent variable values, or that the (unknown) phenomena that caused the spurious result are atypical so that, for the purposes of fitting a general model applicable to most situations, the data point can be deleted.

To determine terms to be included in the model, a series of stepwise regressions were performed with entry significance level equal to 0.10 and required a significance level equal to 0.025 to stay in the model. At each step, the studentized residuals and DIFFITS statistics were examined for identifying spurious results as discussed above. Once a set of data points was determined to use in the regression, the independent variables and terms in the model were determined using a stepwise regression, using p-value of about 0.05 to stay in the model. Once the terms to be included in the model were determined, to estimate the parameter coefficient values for the selected terms in secondary model together with their error covariance matrix, a multivariate, mixed-effect, linear model with a nested error structure was used. The nested error structure reflects the between-experimental combination and

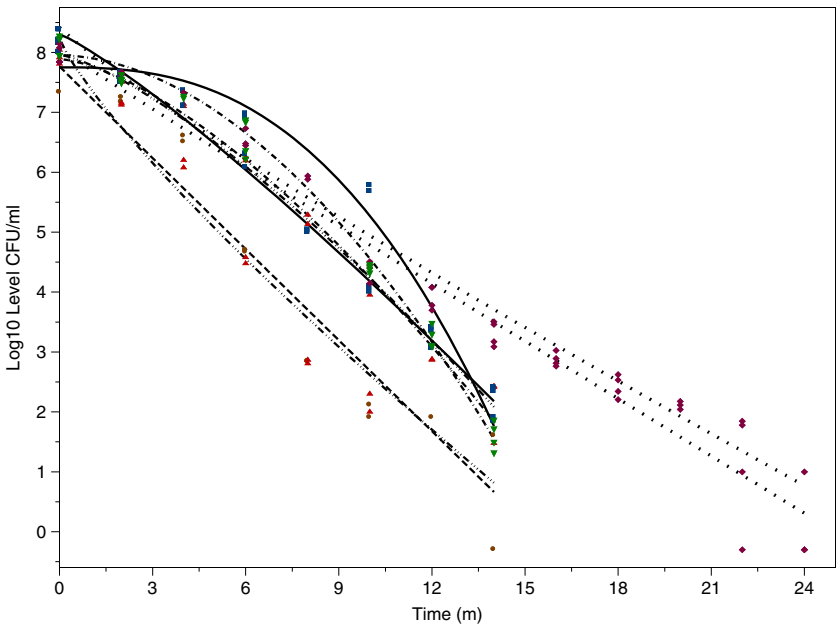

Fig. 3 Plot of observed log of levels and predicted survival curves for temperature=65 °C, salt=2.5 %, SPP=0.3 %, and NaL=2.5 %

the within experimental-combination variance components. This error structure introduces 6 more parameters into the model because there are two correlated dependent variables from the Weibull function.

Maximum likelihood estimation for estimating parameter values was used, using PC SAS® version 9.2. Graphs were constructed using S-PLUS ® version 8.0.

Results and Discussion

Primary Model Our statistical analysis indicated that the Weibull model provided a better fit to the data than that of the linear model. For the 85 survival curves, 52 had estimated value of γ—the shape parameter of the Weibull function—that were less than 1 (implying a convex survival curve), with 27 of them significant at better than the 0.05 two-sided significance level, and 17, at better than the 0.01 two-sided significance level. In the other direction, 33 had values that were greater than 1 (implying a concave survival curve), with 23 of them significant at better than the 0.05 two-sided significance level, and 15, at better than the 0.01 two-sided significance level. The root mean square of the 85 estimated terms, σ_r, for the linear model was 0.52; for the Weibull model, it was 0.34. The likelihood ratio statistic when assuming γ is not equal to 1 versus when assuming γ is equal to 1 was about 570.6, which over the 85 experiments is highly significant (p value$\approx 10^{-60}$). Using the Weibull model, the absolute values of residuals of the predictions of the plate counts at each time (the sum of the duplicate plate counts) were all less than 13.6; the largest was associated with a data point with observed 314 CFU and predicted CFU of 327.6. The largest chi-square test value was 6.5, associated with a data point with observed CFU of 2 and predicted CFU of 0.397. The median chi-square value was 0.0156. Based on these results, we assumed a primary model based on the Weibull function. Thus, our model does not provide D values that are usually associated with inactivation kinetics. In this regard, our model agrees with another model that predicts inactivation kinetics of *L. monocytogenes* (Schultz et al. 2006).

Figure 3 presents the observed data and predicted survival curves for all 9 experiments conducted at a temperature of 65 °C, NaCl=2.5 %, SPP=0.3 %, and NaL=2.5 %. The data points below zero are those observations for which no colony forming units were found for the sample. The individual curves fit the data well. However, there were large differences among the curves. These large differences (between survival curves differences) create uncertainty of predicting lethalities that our model accounts for.

Selection of Secondary Model Parameters Figure 4 presents graphs of the natural log of γ (left graph) and natural log of θ (right graph), versus temperature, with linear OLS regression lines. One data point (73.9 °C, NaCl=0 %, SPP=0 %, and NaL=0 %) is not shown because the estimated values of $\ln(\theta)$ and $\ln(\gamma)$ were estimated to be −14 and −2, respectively. This data point is clearly an outlier: the corresponding values of $\ln(\theta)$ and $\ln(\gamma)$ for its replicate experiment were −2.87 and −0.21, respectively. Thus, we deleted that data point from the remainder of the analysis. Both $\ln(\theta)$ and $\ln(\gamma)$ appear to be linear with temperature, the latter, though, to a lesser degree.

We performed simple correlation analysis, holding three of the four design variables constant, and computing Spearman correlations of the fourth variable with the dependent

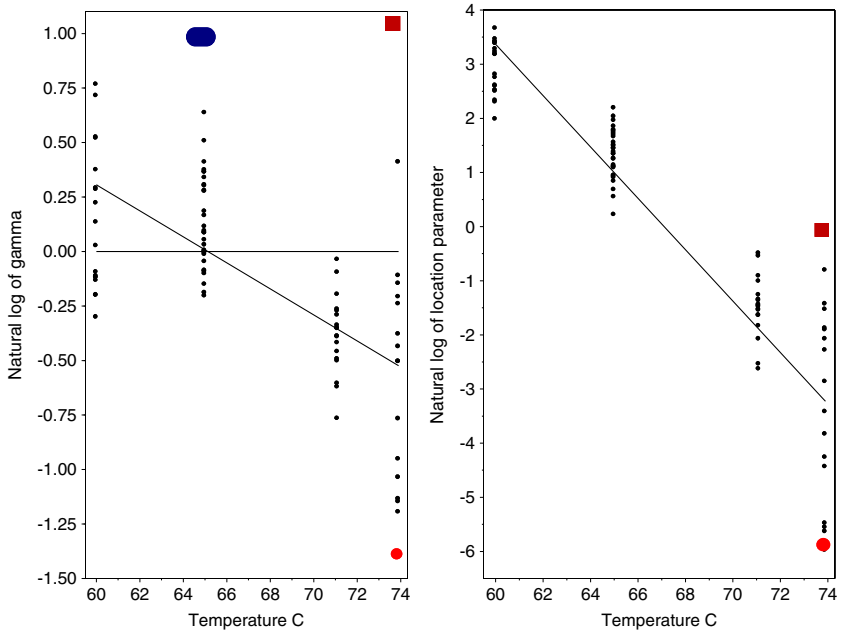

Fig. 4 Plots of primary model Weibull survival curve parameter estimates (θ, γ) on natural log scale versus temperature. One data point not shown because the estimate of $\ln(\theta)$ was equal to about −14. Other specially marked data points were deleted from the analysis used to develop the model

Table 2 Estimated parameter values for secondary model (Eq. 13) used to estimate parameter values (θ, γ) for Weibull primary survival model for arbitrary temperature, salt, SPP, and NaL values within experimental ranges

Variable Term Type	θ Intercept	θ T-60	θ $(T\text{-}60)^2$	θ (T-60) Salt	θ (T-60) SPP	θ Salt NaL	γ Intercept	γ $(T\text{-}60)^2$	γ Salt	γ (T-60) SPP
Estimate	2.815	−0.338	−0.008	0.031	−0.185	0.038	0.010	−0.002	0.096	−0.083
StdErr	0.133	0.033	0.002	0.005	0.041	0.012	0.080	0.001	0.026	0.020
θ	1.000	−0.480	0.236	0.134	−0.109	−0.457	0.449	−0.235	−0.095	−0.157
θ*(T-60)	−0.480	1.000	−0.814	−0.308	−0.158	0.081	0.141	−0.007	−0.180	−0.003
θ*(T-60)*	0.236	−0.814	1.000	−0.011	−0.169	0.048	−0.090	0.409	−0.011	−0.245
θ*(T-60)*	0.134	−0.308	−0.011	1.000	0.022	−0.341	−0.493	0.018	0.633	0.008
θ*(T-60)*	−0.109	−0.158	−0.169	0.022	1.000	−0.035	−0.133	−0.474	−0.000	0.787
θ*Salt*N	−0.457	0.081	0.048	−0.341	−0.035	1.000	−0.110	0.010	0.144	−0.009
γ	0.449	0.141	−0.090	−0.493	−0.133	−0.110	1.000	−0.268	−0.777	−0.172
$\gamma(T\text{-}60)^2$	−0.235	−0.007	0.409	0.018	−0.474	0.010	−0.268	1.000	0.033	−0.600
Salt*γ	−0.095	−0.180	−0.011	0.633	−0.000	0.144	−0.777	0.033	1.000	0.002
(T-60)*SP	−0.157	−0.003	−0.245	0.008	0.787	−0.009	−0.172	−0.600	0.002	1.000

Variable name prefixed with θ indicates variables used for predicting values of ln(θ), and similarly for γ, for estimating ln(γ). First two rows are estimated values of the parameters of the secondary model and their standard errors; the last 10 rows is the error correlation matrix

variables, ln(γ) and ln(θ) over the different combinations. Correlations of the estimated values of θ and salt were all positive indicating a strong general effect of salt on inactivation kinetics. For the SPP and NaL variables, many correlations were negative; for the SPP variable, the correlations were mostly negative for temperatures of 71 °C and 73.9 °C. This suggests a possible interactive impact of SPP and temperature on the inactivation kinetics. This analysis though suggests that, besides temperature, only salt has a clear simple effect on the inactivation kinetics of *L. monocytogenes*.

To determine a secondary model, we first performed stepwise regressions using all the data, except the one data point identified above. We used temperature minus 60 (T−60) instead of temperature as an independent variable, l. For the dependent variable, ln(γ), one data point with experimental combination (65 °C, 2.5 %, 0.30 %, 2.5 %) identified in Fig. 4 as the ellipse, the studentized residual was 3.18 and DFFITS value of 1.60. The value of γ for this experiment was 2.62; the value for the replicate experiment with the same experimental conditions was 1.18. The difference on the logarithmic scale, in absolute value, between these two values was the largest among all experimental conditions. Consequently, we deleted this data point from all further analysis.

Using the remaining 83 data points, from the stepwise regression with dependent variable, ln(θ), the data point with experimental combination at (73.9 °C, 4.5 %, 0.5 %, 0.0 %) —the identified data point in the lower right (circle) at 73.9 °C in both graphs of Fig. 4 had a studentized residual of −4.91. This data point also had an exceptionally large, in absolute value, influence statistic, DFFITS, value of −2.82. Deleting this data point and running the regression for predicting ln(γ) with the remaining 82 data points, the data point at (73.9 °C, 4.5 %, 0.0 %, 4.5 %) in the upper right (square) in both graphs of Fig. 4 had a studentized residual 3.05 and influence statistic, DFFITS, value of 1.73. Consequently, this data point was also deleted, leaving us with 81 data points.

The stepwise regressions with dependent variables ln(θ) and ln(γ) using the 81 data points had no large studentized residuals, but, for the dependent variable ln(θ), there was one data point at (73.9 °C, 4.5 %, 0.5 %, 0.0 %) with large DFFITS statistic of 1.2. The

Table 3 Estimates of random between and within experimental combinations standard deviations and correlations

Between Std dev ln(θ)	Between Std dev ln(γ)	Corr ln(θ), ln(γ) Between	Within Std dev ln(θ)	Within Std dev ln(γ)	Corr ln(θ), ln(γ) Within
0.376	0.192	0.764	0.326	0.157	0.874

Table 4 Estimated expected time to achieve 5 or 6 log reduction of *L. monocytogenes* for selected temperature, salt, SPP, and NaL values, with 90 % lower and upper confidence bounds

Temp (°C)	Salt (%)	SPP (%)	NaL (%)	Expected Time (m) 5 log Lethality	Lower 90 % Bound 5 log Lethality	Upper 90 % Bound 5 log Lethality	Expected Time (m) 6 log Lethality	Lower 90 % Bound 6 log Lethality	Upper 90 % Bound 6 log Lethality
60	0	0.0	0	82.1	52.0	130	98.4	56.5	171
60	4	0.0	0	49.4	37.8	64.5	55.8	40.7	76.5
66	4	0.0	2	15.2	12.1	19.0	17.3	13.1	23.0
66	4	0.5	2	12.2	8.88	16.8	14.5	9.75	21.5
72	0	0.0	0	0.82	0.40	1.66	1.05	0.43	2.56
72	4	0.5	0	1.58	0.75	3.34	2.11	0.84	5.30
72	4	0.5	4	2.92	1.41	6.04	3.89	1.58	9.57

impact of excluding this data point for predicting values for θ and γ is not large, because the stepwise regressions for both ln(θ) and ln(γ) selects the same independent variables whether this data point is or is not included and the differences of values of the estimated coefficients of these variables are small. This data point was not deleted in subsequent analyses.

For the dependent variable, ln(θ), the 5 variables selected were: Temp-60, $(\text{Temp-60})^2$, Salt*(Temp-60), Spp×(Temp-60), and Salt*NaL. For the dependent variable, ln(γ), the 3 variables selected were $(\text{Temp-60})^2$, Salt and (Temp-60)*SPP. The NaL variable occurs only interacting with salt. Adding a linear term of NaL to the model was statistically not significant with two-sided p-value equal to 0.42, based on the likelihood ratio test. When not using data for 73.9 °C, NaL does not enter into the model for ln(θ) as a statistically significant variable in the stepwise regression; however, the interaction term (Salt)(NaL) does enter the model as a significant variable.

Secondary Model Our secondary model (Eq. 3) is:

$$\begin{matrix} \widehat{\ln(\theta_{kj})} \\ \widehat{\ln(\gamma_{kj})} \end{matrix} = \begin{matrix} h_1 + h_2(T-60) + h_3(T-60)^2 + h_4(\text{Salt})(T-60) + h_5(\text{SPP})(T-60) + h_6(\text{NaL})(\text{Salt}) + \varepsilon_{\theta k} + \varepsilon_{\theta kj} \\ h_7 + h_8(T-60)^2 + h_9(\text{Salt}) + h_{10}(T-60)(SPP) + \varepsilon_{\gamma k} + \varepsilon_{\gamma kj} \end{matrix} \quad (3)$$

where $\widehat{\theta_{kj}}$ and $\widehat{\gamma_{kj}}$ are the estimated values of the parameters θ and γ of the Weibull survival curve for the *j*th replicate of the *k*th survival experimental combination; h_i, i=1, …, 10 are unknown parameters whose values are estimated from the multivariate regression; ($\varepsilon_{\theta k}$, $\varepsilon_{\gamma k}$) are assumed random effects for the *k*th experimental design combination, k=1, …, 43, assumed to be bivariate normal distributed with 2×2 covariance matrix, Σ_b, and expected values of 0; and similarly, ($\varepsilon_{\theta kj}$, $\varepsilon_{\gamma kj}$) are assumed random effects for the *j*th replicate, j=1,2, within the *k*th experimental design assumed to be independent of ($\varepsilon_{\theta k}$, $\varepsilon_{\gamma k}$) and bivariate normal distributed with 2×2 covariance matrix, Σ_w, and expected value of 0.

Table 2 provides the estimated values of h_i, i=1, … , 10, together with their standard errors and correlation matrix (below the double line). Table 3 provides the estimated covariance matrices, Σ_b, and Σ_w. The likelihood ratio statistic for testing the significance of Σ_b, was 37.3 which, based on 3 degrees of freedom, is highly significant (p value=3.9×10^{-8}).

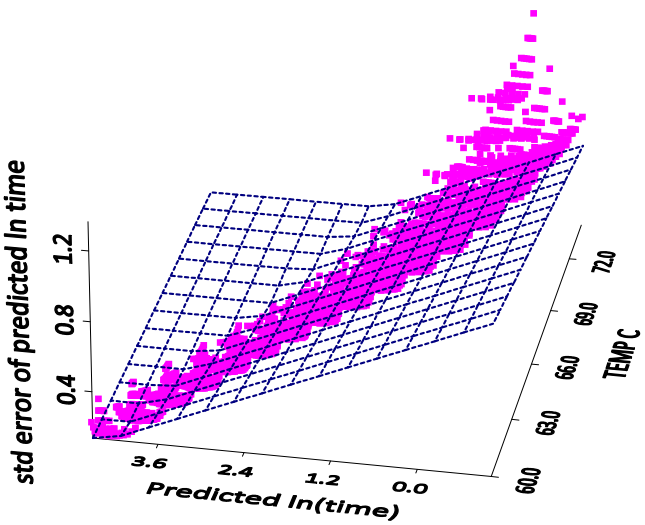

Fig. 5 Plot of standard error of prediction of natural log of time to obtain 5 log lethality

Our model provides estimates of expected lethalities for given time, temperature, salt, SPP, and NaL values. Often, there is a specified lethality, L, that must be obtained to ensure a safe product or to satisfy some regulatory requirement. For this problem, one needs to solve for ln(t), given values of θ and γ. For the Weibull function, the solution for the needed time, t, is:

$$\ln(t) = \ln(L) \Big/ e^{\ln(\gamma)} + \ln(\theta). \quad (4)$$

We estimate the standard errors of the estimated expected natural logarithm of time using the linear terms of the Taylor series expansion, relative to the transformed variables ln(γ) and ln(θ). From these we calculated 90 % upper and lower confidence bounds for the estimated times.

Predicted expected times, together with their 90 % upper and lower confidence bounds for selected temperatures, NaCl, SPP, and NaL values for lethalities equal to 5 or 6 log reduction are presented in Table 4. After temperature, NaCl has the largest impact on the needed time. Figure 5 presents a 3D graph of the standard error of predictions versus temperature and the predicted natural log of the time. For high temperatures, the standard error of the predicted lethalities is large causing relatively wide confidence intervals. The large magnitude of the confidence intervals suggests that more experiments are needed for high temperatures.

Conclusion

The model presented in this paper can be used as an aid in designing lethality treatments meant to control the presence of *L. monocytogenes* in ready-to-eat product. The results indicate that temperature and NaCl have a large impact on the inactivation kinetics of *L. monocytogenes*, while sodium lactate (NaL) has an impact in the presence of NaCl. Our model takes into account large degrees of uncertainty in predictions of lethalities at given conditions. The information in this paper permits users to incorporate this uncertainty for predicting lethalities to help ensure lethalities at targeted levels.

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